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Polyphenolic Compounds of a New Biologically Active Extract from Immortelle Sandy Flowers (*Helichrysum arenarium* (L.) Moench.)

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Abstract—The chemical composition of a new bioactive extract from immortelle sandy (*Helichrysum arenarium* (L.) Moench.) was investigated. Naringin, its soluble aggregate, prunin, quercetin, apigenin, naringenin, apigenin 5-*O*-glucoside, and isosalipurposide were found among the flavonoids. The tested immortelle sandy extract was shown to contain known dimers, trimers, or more complex aggregates. The compounds had similar spectral but substantially different chromatographic characteristics, which can be used for their identification based on the corresponding data on flavonoids glycosylated at different positions and/or carbohydrates of various complexities, which are also characterized by similar absorption spectra and different retention times. It was found by molecular absorption spectroscopy that the immortelle extract contained 73.48 mg of flavonoids relative to rutin or 17.94 mg relative to quercetin per 1 g of dry extract weight, which corresponded to 20.99 and 5.13%, respectively. The immortelle extract obtained by the method proposed displayed antitumor activity against the transplanted sarcoma 45 and had a beneficial effect on animals in general.

Keywords: chemical composition, flavonoids, Helichrysum arenarium (L.) Moench., HPLC

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INTRODUCTION

Flavonoids are a group of polyphenol compounds with a wide spectrum of pharmacological activities (antimicrobial, choleretic, hepatoprotector, etc.) [1, 2]. One of the promising flavonoid sources is herbal materials, particularly, immortelle sandy flowers (*Helichrysum arenarium* (L.) Moench), a perennial plant of the Asteraceae family [3–7]. Currently, immortelle sandy flowers are used in medicine as spasmolytic, choleretic, bactericide, and anti-inflammatory agents. These properties are assigned to the presence in the extract of polyphenol compounds, flavonoids, in particular, the total content of which in the inflorescence varies from 6.18 ± 0.43 to 6.46 ± 0.65% depending on the inhabitation of *H. arenarium* cenopopulation [8]. It was found that the extracts of immortelle sandy flowers contained both glycosylated and nonglycosylated flavonoids, the question about prevailing flavonoid being open [9–12].

Different extractions methods for the same plant raw material can lead to biologically active compounds of different chemical composition and properties [13]. It was shown previously that the extraction of biologically active compounds with 96% ethanol followed by evaporation at 55–60°C, dissolution of the extract in water, and additional purification from hydrophobic components (chlorophyll, essential oils, and tannins) with chloroform [14] enhanced the biological activity of aqueous solutions of dry extracts on the one hand and considerably reduced their toxicity on the other hand [15–18]. An increase in the biological activity of the extracts obtained by this approach with the drug hedge hyssop (*Gratiola officinalis*) as an example [17, 18] is associated with an increase in the flavonoid yield. Therefore, it seems topical to obtain extracts from other herb materials using this approach and study their biological activity, primarily, that of immortelle sandy flowers. It has been found that the immortelle extract obtained by the method described below manifested anticancer properties against the transplanted sarcoma 45 and had a beneficial effect on animals in general [18].

The goal of our work was the analysis of components of a new biologically active mixture from immortelle sandy flowers (*Helichrysum arenarium* (L.) Moench.) using electron spectroscopy and high performance liquid chromatography.

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EXPERIMENTAL

Collection of the material. The raw material (flowers) was collected in the Lysogorsk region of Saratov oblast and the neighborhood of Ataevka village in July of 2013. Dry immortelle sandy leaves and flowers were preliminarily ground to achieve a more exhaustive extraction.

Extraction. Ground dry material (10 g) was placed in a round-bottom flask, 96% ethanol (100 mL) was added, and the mixture was refluxed on a water bath for 15 min. The alcohol extract was evaporated to dryness in a thermostat at 55–60°C. Warm distilled water (40–50°C, 8 mL, 4/5 of the total volume) was added to the residue, thoroughly mixed, and chloroform (2 mL, 1/5 of the total volume) was added. The mixture was shaken to a homogenous emulsion, cooled to room temperature, and centrifuged at 1500 rpm to separate completely the chloroform fraction containing nonpolar impurities and the water fraction with target products. The separation of the water fraction followed by drying in the Petri dish resulted in a dry residue of target products, which allowed the calculation of accurate dosages for in vitro and in vivo experiments as well as long term storage of the extract.

Differential absorption spectroscopy. The studies were performed on a UV-1700 spectrophotometer (Shimadzu, Japan) in the Center of Collective Use at the Chernyshevski Chemistry Institute, Saratov State University. Quartz cuvettes with absorption layer thickness of 1 cm were used for the measurements. For plotting calibration curves, 1 mg/mL working solutions of rutin and quercetin complexes with a solution of 5% aluminum chloride in 96% ethanol were prepared from the flavonoid solutions. The proper volume of the solution was taken out, 5% $AlCl_3$ (1 mL) was added, and ethanol was added to the volume of 5 mL. Absorption spectra of the solutions were measured in 30 min within the range of 250 to 600 nm using 5% $AlCl_3$ in 96% ethanol as a reference solution.

High performance liquid chromatography (HPLC). For the analysis of polyphenol compounds the Dionex Ultimate 3000 HPLC system (Thermo Scientific, United States) equipped with a Luna 5μ C18(2) column (150 × 4.60 mm) (Phenomenex, United States) and a diode array detector were used. HPLC analysis was performed in the Center of Collective Use with the physicochemical biology and nanobiotechnology "Simbios" equipment of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences. The dry extract was dissolved in a 1 : 1 acetonitrile–water (of the MilliQ grade) mixture (0.5 mL) adjusted to pH 2.5 with 50% H_3PO_4. The extracts were chromatographed in a gradient mode. Component A of the mobile phase was 100% MeCN; component B, water (of the MilliQ grade) adjusted with 50% H_3PO_4 to pH 2.5; 0–10 min, 15% A, 85% B; 10–19 min, 15 → 70% A, 85 → 30% B; 19–20 min, 70% A, 30% B; 20–22 min, 70 → 15% A, 30 → 85% B; 22–25 min, 15% A, 85% B; the flow rate, 1 mL/min; the sample volume, 20 μL; the column temperature, 30°C; detection from 200 to 600 nm and integration at 342 nm.

Chromatograms as well as absorption spectra in the UV and visible ranges were obtained for each component using HPLC.

As reference standards of flavonoids, rutin hydrate (≥94%, Sigma-Aldrich, United States), quercetin dihydrate (97%, Alfa Aesar, England), naringin (≥95%, Sigma-Aldrich, United States), apigenin (≥97%, Sigma-Aldrich, United States), naringenin (≥95%, Sigma-Aldrich, United States), and prunin obtained by partial acid hydrolysis of naringin were used.

RESULTS AND DISCUSSION

For the extraction of *Helichrysum arenarium* (L.) Moench flowers we used the previously developed method ensuring a high yield of flavonoids, which was demonstrated with the drug hedge hyssop. In addition to the increase in the flavonoid yield, this method provided a decrease in the toxicity of the extract for in vitro and in vivo experiments [17, 18]. Also, a higher alcohol concentration in the extracting agent, if compared with the previously used methods, allowed a lower extraction temperature.

Since the raw plant material contained not only water soluble but also hydrophobic components (for example, chlorophyll, essential oils, and tannins), the latter were additionally extracted with chloroform.

Polyphenol compounds in immortelle sandy flowers were represented by both glycosylated (isosalipurposide, salipurposide, A and B isomers of helihrizin, prunin, and apigenin 5-*O*-glucoside) and nonglycosylated (aglycones) (apigenin, kaempferol, and naringenin) forms. It is known that flavonoid glycosides are better extracted with 70–80% alcohol, whereas aglycones, with 90–96% alcohol. It can be expected that the treatment of the water fraction with chloroform followed by the separation of the organic layer would result in a slight decrease in the aglycone content. However, as we demonstrated by the comparison of the extracts obtained by different methods, with and without the chloroform treatment, it is impossible to achieve the total extraction of nonglycosylated flavonoids with chloroform under the conditions we proposed. Moreover, the apigenin content did not change after the chloroform treatment.

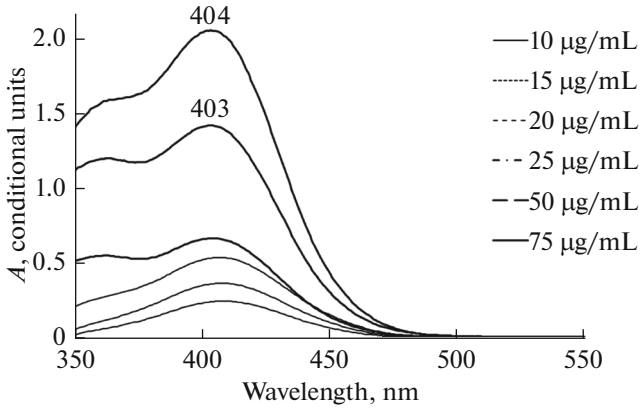
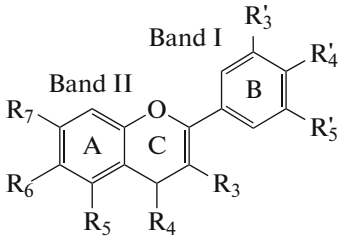


Fig. 1. Absorption spectra of solutions of rutin complexes with aluminum chloride at various concentrations in the range of 350–550 nm. The complex absorption maxima at 404–406 nm. The reaction time of 30 min.



R = H, OH, OMe, OGlyc.

Phenol compounds are known to form specifically colored complexes with ferric and aluminum chlorides. The interaction of phenol-derived compounds with aluminum chloride results in a shift of the absorption maximum in the electronic spectrum. Although the presence of one hydroxyl group is enough for the reaction (the known qualitative reaction for the phenolic hydroxyl group with ferric(III) chloride), the composition of the complexes obtained can be rather complex, because either one flavonoid hydroxyl group or two groups in the case of their favorable steric location can be involved in the coordination. In addition, flavonoids having a carbonyl residue in the C ring can also use it upon coordination [20, 21]. This reaction is often used for spectrophotometric assays of polyphenol compounds in plant extracts.

The method of differential absorption spectroscopy in the UV and visible bands allows the total flavonoid content in the material tested [21, 22]. However, this method has some limitations. First of all, they are related to the lack of a "universal" standard with the same absorption parameters as the tested plant extract. Since as a rule the latter contains flavonoids of different classes, chalcones, flavones, flavonols, etc., which differ in maxima and their intensities (governed by the molecular structure, particularly, by conjugation chains, substituents, etc.), the superposition of absorption spectra of these compounds is a complicated picture. This restriction can be partially removed by the use of the standard for plotting the calibration curve, which would correspond in its structure to the flavonoid or flavonoid class of a particular plant extract most representative in the absorption spectrum at the given wavelength (which would not necessarily correlate with the mass percentage). However, even in this case, the results should be treated with caution.

When comparing the absorption spectra of complexes of quercetin and its glycosylated derivative rutin one can note that the complex formation caused the bathochromic shift of the longer wavelength band I of the B ring chromophore. Also, it is noteworthy that at the wavelength of the longest wavelength maximum the absorption of nonglycosylated quercetin was four times as high if compared with its glycoside rutin at the same concentration.

In the range of ~370–540 nm, differential absorption spectra of aluminum chloride complexes with flavonoids rutin and isosalipurposide contained a wide absorption band at 410–412 nm for rutin and 418 nm for salipurposide respectively [24]. Despite the proximity of maximum positions, the absorption intensity of the isosalipurposide complex was about five times higher. Unfortunately, the concentrations, at which the spectra were recorded, were not reported. However, if we take them equal, the use of a standard when assaying the total flavonoid level in the immortelle sandy extract with chaconne type flavonoids prevailed, approximately a fivefold decrease in this value can be expected. At the same time, according to our data, the absorption of the quercetin complex with aluminum chloride at the same wavelength was about 3 to 4 times higher than that for the similar rutin complex.

We used this approach for the estimation of the total flavonoid level relative to rutin and quercetin. Linear functions were observed within the concentration range of 5 to 100 μg/mL for rutin and 3 to 25 μg/mL for quercetin complexes with the correlation coefficients of 0.9995 and 0.9980 respectively.

The results obtained by differential absorption spectroscopy in the UV and visible ranges are shown in Figs. 1–3. Based on this method the immortelle sandy extract contained 73.48 mg of flavonoids relative to rutin and 17.94 mg relative to quercetin per 1 g of dry extract mass, which is equivalent to 20.99 and 5.13% respectively.

Since the plant extract is a complex mixture of compounds of different nature, for the determination of its composition HPLC with the UV–visible detection is usually used. The gradient method we used allowed the first elution of hydrophilic components (for example, organic acids; particularly, in plant extracts gallic acid and glycosylated flavonoids are most abundant), and then of more hydrophobic compounds, particularly, nonglycosylated flavonoids, in a

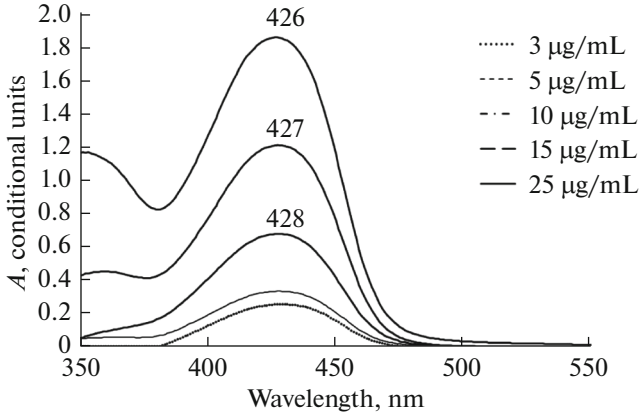


Fig. 2. Absorption spectra of solutions of quercetin complexes with aluminum chloride at various concentrations in the range of 350–550 nm. The complex absorption maxima at 426–428 nm. The reaction time of 30 min.

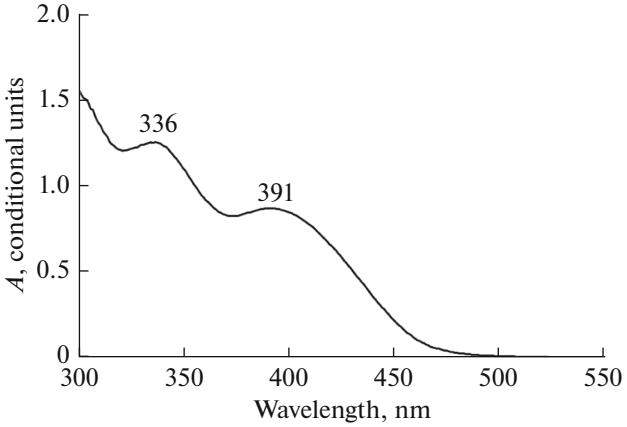


Fig. 3. Absorption spectrum of immortelle sandy extract treated with aluminum chloride at various concentrations in the range of 300–550 nm. The complex absorption maxima at 336 and 391 nm. The reaction time of 30 min.

rather short analysis time (the total time of about 25 min). Obviously, the longer was flavonoid carbohydrate chain, the higher was its hydrophilicity. Flavonoid monoglycosides (flavonoids glycosylated with a monosaccharide) are more hydrophilic than aglycones, whereas diglycosides (called disaccharide monoglycosides in some publications) in its turn are more hydrophilic than monosaccharide flavonoids, because under these conditions for an individual flavonoid the retention times of the proper diglycosylated and monoglycosylated derivatives and aglycone increased. The retention time is affected not only by the aglycone structure but presumably by the glycosylation position too. It was clearly seen when compar-

Chromatographic properties of the extracts treated and untreated with chloroform

Component of the mixture	Chloroform-treated			Chloroform-untreated		
	retention time, min	area, mau × min	relative amount*, %	retention time, min	area, mau × min	relative amount*, %
1 (component 2 aggregate)	1.758	6.0749	2.54	1.755	3.8802	3.03
2	1.838	24.4143	10.22	1.842	13.7556	10.74
3	3.727	1.7440	0.73	3.728	0.3926	0.31
4	14.005	6.4077	2.68	14.005	4.5930	3.59
5 (naringin aggregate)	14.702	38.5065	16.11	14.703	27.3803	21.39
6 (naringin)	14.850	23.9304	10.01	14.855	17.8020	13.91
7 (prunin)	14.997	13.5123	5.65	15.007	8.5991	6.72
8 (apigenin 5-*O*-glucoside)	15.128	3.9334	1.65	15.130	2.1801	1.70
9	15.260	7.8981	3.31	15.302	2.6109	2.04
10 (isosalipurposide aggregate)	15.585	35.1308	14.70	15.638	8.9906	7.02
11 (isosalipurposide)	15.737	40.4213	16.92	15.745	10.1033	7.89
12	15.872	2.0501	0.86	15.873	0.6945	0.54
13	16.408	5.1092	2.14	16.413	0.9723	0.76
14 (quercetin)	16.827	1.6096	0.67	16.830	1.6495	1.29
15	16.917	0.5472	0.23	16.923	0.4174	0.33
16	17.232	0.7867	0.33	17.233	0.0257	0.02
17 (apigenin)	17.763	18.3570	7.68	17.765	17.4382	13.62
18 (naringenin)	17.972	1.8171	0.76	17.973	2.9560	2.31

* Relative amount of the mixture component calculated as a ratio of its peak area to the summary of peak areas of all components and expressed in %.

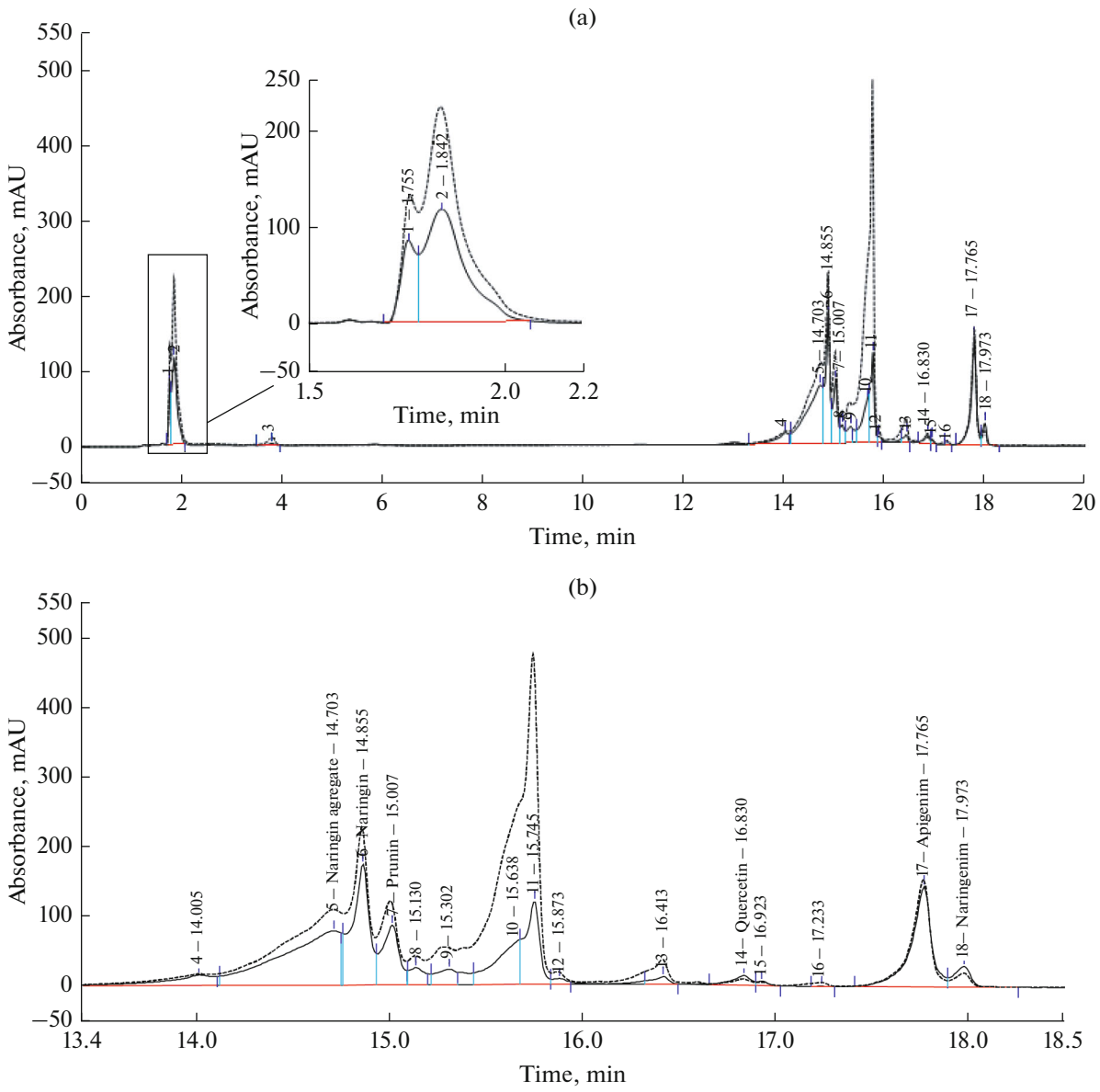


Fig. 4. Full chromatogram of (a) immortal sandy extract and (b) chromatogram fragment from 13.50 to 18.50 min. A solid line corresponds to the chloroform-treated extract and the dotted line, to chloroform-untreated extract.

ing the retention times of quercetin, naringenin, and their mono- and diglycosides. For example, the retention time of quercetin diglycoside, rutin, quercetin 3-*O*-rhamnoglucoside, was 1.84 min, whereas that of quercetin, 16.83 min. The retention time of naringenin, which is less hydrophilic if compared with quercetin, was 17.87 min. Naringenin bears a saturated C ring and lacks a substituent at position 3, which probably supports a kind of a molecule hydrophobic site. At the same time, the difference between the retention times of naringenin monoglycoside (naringenin 7-*O*-glucoside, pruning) and diglycoside (naringenin 7-*O*-rhamnoglucoside, naringin) is not so large (15.00 and 14.80 min respectively). A slight difference in the hydrophobicity of these molecules can be explained by glycosylation at position 7, because their hydrophobic regions are not shielded by hydrophilic carbohydrate fragments as it was for quercetin 3-*O*-glycosides.

Using HPLC we found 18 major components of the immortelle sandy extract (table, Fig. 4). The structures of some of them could be ascribed. Particularly, components 6, 7, 14, 17, and 18 with retention times of 14.85, 15.00, 16.83, 17.76, and 17.97 min proved to be naringin, prunin, quercetin, apigenin, and naringenin. The identity of the components found with the standards was confirmed by the standard addition method. UV spectra of the components found and the stan-

dards were completely identical. It is noteworthy that the spectrum of component 5 was very similar to that of component 6 (naringin), although the intensity of the former was somewhat lower. The naringin aggregated form is known to remain soluble with the absorbance identical or close to that of the monomeric form [25]. Some amount of the soluble naringin aggregated form was observed on the chromatogram of the standard of this flavonoid. Therefore, we assumed that component 5 was a soluble naringin aggregate.

The retention time of component 2 coincided with that of the rutin standard (1.84 min). However, since the standard and this extract component differed in the positions of their UV absorption maxima, we did not assign component 2 to rutin. The UV spectra of peaks 1 and 2 were similar, but the intensity of component 1 was somewhat lower, which supported a hypothesis on the aggregate nature of component 1 as it was observed for naringin.

Since glycosylation did not nearly effect on the positions of absorption maxima, the similarity of UV spectra for apigenin and component 8 allowed a suggestion that component 8 with the retention time of 15.13 min could be apigenin glycoside, presumably, 5-*O*-glucoside.

Component 11 was characterized by the largest peak area with one of the longest wavelength absorption maxima (~368 nm) and based on the published data it can be assigned to isosalipurposide (372 nm [9]). Thus, since UV spectra of components 10 and 11 only differed in the absorption intensity, component 10 can be an aggregate of this flavonoid.

As follows from Fig. 4 and the table, the treatment of the extract with chloroform resulted in a considerable decrease in the absolute content of naringin and its soluble aggregate form as well as salipurposide and the respective aggregate form. If for the latter the relative amount calculated by the peak areas was reduced at least by half, the content of naringin and its aggregate form even slightly increased. The absolute amount of quercetin and apigenin prior to and after the chloroform treatment remained nearly the same, but due to a decrease in the portions of the other components their relative amount in the extract after the chloroform treatment increased nearly twice.

CONCLUSIONS

In the biologically active composition of flavonoids isolated from immortelle sandy we found naringin and its aggregated form, prunin, quercetin, apigenin, and naringenin as well as apigenin 5-*O*-glucoside and isosalipurposide. Using the data of molecular absorption spectroscopy, we found 73.48 mg of flavonoids relative to rutin and 17.94 mg relative to quercetin in 350 g of the dry extract. Thus, the percentage of flavonoids was 5.13 to 20.99% depending on the standard used.

The extract obtained from immortelle sandy by the method described in this work can be recommended for further studies of its biological properties.

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